INFECTION AND IMMUNITY, May 2005, p. 2698–2703 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.2698–2703.2005

Pertussis Toxin and Adenylate Cyclase Toxin Provide a One-Two Punch for Establishment of *Bordetella pertussis* Infection of the Respiratory Tract

Nicholas H. Carbonetti,* Galina V. Artamonova, Charlotte Andreasen, and Nicholas Bushar

Department of Microbiology and Immunology, University of Maryland Medical School, Baltimore, Maryland 21201

Received 22 October 2004/Returned for modification 4 January 2005/Accepted 10 January 2005

Previously we found that pertussis toxin (PT), an exotoxin virulence factor produced by *Bordetella pertussis*, plays an important early role in colonization of the respiratory tract by this pathogen, using a mouse intranasal infection model. In this study, we examined the early role played by another exotoxin produced by this pathogen, adenylate cyclase toxin (ACT). By comparing a wild-type strain to a mutant strain (ΔCYA) with an in-frame deletion of the *cyaA* gene encoding ACT, we found that the lack of ACT confers a significant peak (day 7) colonization defect (1 to 2 $\log_{10}$). In mixed-infection experiments, the ΔCYA strain was significantly outcompeted by the wild-type strain, and intranasal administration of purified ACT did not increase colonization by ΔCYA. These data suggest that ACT benefits the bacterial cells that produce it and, unlike PT, does not act as a soluble factor benefiting the entire infecting bacterial population. Comparison of lower respiratory tract infections over the first 4 days after inoculation revealed that the colonization defect of the PT deletion strain was apparent earlier than that of ΔCYA, suggesting that PT plays an earlier role than ACT in the establishment of *B. pertussis* infection. Examination of cells in the bronchoalveolar lavage fluid of infected mice revealed that, unlike PT, ACT does not appear to inhibit neutrophil influx to the respiratory tract early after infection but may combat neutrophil activity once influx has occurred.

Bordetella pertussis, a gram-negative bacterial pathogen of the human respiratory tract, secretes at least two protein toxins, pertussis toxin (PT) and adenylate cyclase toxin (ACT), that are important virulence factors in mouse models of infection. PT is an AB_5 toxin that is uniquely produced by *B. pertussis*. It comprises an enzymatically active A subunit (S1) that ADP ribosylates the alpha subunit of heterotrimeric Gi proteins in mammalian cells (17, 22) and a B heteropentamer that binds unidentified glycoconjugate receptors on cells (1, 32). ADP ribosylation of G proteins by PT causes a wide range of effects on signaling pathways in mammalian cells (25) and is responsible for the systemic symptoms of pertussis disease, such as lymphocytosis, insulinemia, and histamine sensitivity (21, 23, 24). Recently, by comparing a wild-type strain to a mutant strain with an in-frame deletion of the genes encoding PT (ΔPT), we found that PT is an important colonization factor for *B. pertussis* lower respiratory tract infection and that PT plays an early role in this host-pathogen interaction, including delaying the recruitment of neutrophils to the site of infection (2). We also found that PT acts as a soluble factor that can enhance *B. pertussis* respiratory tract colonization, even when administered 14 days prior to bacterial inoculation (2). PT also suppresses serum antibody responses to *B. pertussis* after respiratory tract infection (3) and may play multiple immunosuppressive roles in the host-pathogen interaction.

ACT is a single 177-kDa polypeptide with an N-terminal adenylate cyclase domain and a larger C-terminal domain responsible for hemolysis and for entry of ACT into mammalian cells (6). ACT binds specifically to phagocytic cells by interacting with the CD11b/CD18 integrin receptor (12) but can also penetrate lipid bilayers in the absence of this receptor (20, 27). Within cells ACT is activated by calmodulin to generate cyclic AMP (33), the accumulation of which is thought to be the primary mechanism of intoxication (4, 8). ACT inhibits neutrophil functions, including phagocytosis, oxidative burst, and chemotaxis (4, 5, 29), and also induces apoptosis in macrophages both in vitro and in vivo (11, 19). ACT is considered to be an important virulence factor for *B. pertussis*. A Tn*5* mutant of strain Tohama I deficient in ACT production was significantly reduced in virulence in neonatal and infant mice (7, 10, 30, 31) and various different ACT mutants of strain 18323 were defective for respiratory tract colonization of 3- to 4-week-old mice (18). Antibodies to ACT protected infant mice against lethal respiratory challenge with *B. pertussis* (13) and promoted the phagocytosis of *B. pertussis* by human neutrophils (28). A study comparing PT-deficient (ΔPT) and ACT-deficient (ΔACT) mutants in intranasally infected infant mice concluded that ACT, but not PT, was critical for initial colonization of the respiratory tract and that both toxins were necessary for lethality in this model, suggesting that PT played a later role in this interaction (7, 30).

ACT is secreted by *B. pertussis* via the type I pathway encoded by the *cyaB*, *cyaD*, and *cyaE* genes (6), but ACT was first discovered as a bacterial-cell-associated activity (15). Recent studies have shown that ACT associates with filamentous hemagglutinin on the surface of *B. pertussis* (34) but that newly secreted toxin rather than this cell-associated toxin is responsible for intoxication of a macrophage cell line in culture (9). Therefore, it was unclear whether ACT acts as a soluble toxin (like PT) or a cell-associated toxin during infection and colonization of the respiratory tract. We sought to answer this

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Maryland School of Medicine, 655 W. Baltimore St., BRB13-009, Baltimore, MD 21201. Phone: (410) 706-7677. Fax: (410) 706-2129. E-mail: ncarbone@umaryland.edu.

question and to compare the roles of the two toxins in the early respiratory tract events of this host-pathogen interaction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. pertussis* background strain used in this study was a streptomycin- and nalidixic acid-resistant derivative of Tohama I (16). The wild-type strain (WT) was the parental strain that emerged from the conjugation experiment in which the ΔPT strain was constructed (2). The ΔPT and ΔCYA derivatives were constructed as previously described (2, 3). The ΔPT ΔCYA double mutant was derived by introducing the *cyaA* deletion into the ΔPT strain by conjugation and allelic exchange as previously described (3). *B. pertussis* strains were grown on Bordet-Gengou agar (Difco) plates containing 15% defibrinated sheep blood and the following antibiotics at the indicated concentrations where necessary: streptomycin, 400 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; or gentamicin, 10 μg ml^{-1}. *Escherichia coli* strains used were DH10B (Invitrogen) for standard cloning experiments and S17.1 (26) for conjugation with *B. pertussis*, and these were grown on LB agar plates containing gentamicin at 10 μg ml^{-1} where necessary.

Mouse infection. Six-week-old female BALB/c mice (Charles River Laboratories) were used for infection experiments. Inocula were prepared, and intranasal inoculation was performed as previously described (2). At the indicated time points, mice were sacrificed by carbon dioxide inhalation, their lower respiratory tract (trachea plus lungs) was removed and homogenized in 2 ml of phosphate-buffered saline, and dilutions were plated on Bordet-Gengou-blood agar plates with streptomycin. Four days later the number of CFU per respiratory tract was determined. Statistical analysis was performed using a *t* test.

BAL. Mice were sacrificed by carbon dioxide inhalation, and the respiratory tract was exposed by dissection. A small incision was made near the top of the trachea, and a blunt-ended 20-gauge needle was inserted and tied in place with surgical thread around the trachea. Bronchoalveolar lavage (BAL) fluid was obtained by four rounds of filling the lungs with 0.7 ml of phosphate-buffered saline and withdrawing as much of the liquid as possible. BAL samples were centrifuged to pellet the cells, which were resuspended in 1 ml RPMI medium, and aliquots were removed for counting on a hemocytometer and for cytospin centrifugation onto a microscope slide, followed by staining with modified Wright's stain for cell type identification. To determine the number of macrophages and neutrophils in these samples, 100 cells from several microscopy fields were identified.

ACT protein. ACT (a generous gift from Erik Hewlett) was purified from an overexpressing *E. coli* strain, its activity was determined as previously described (8), and it was stored in 8 M urea buffer at −20°C. ACT activity was also confirmed by cytotoxicity for J774.1 macrophage cells, as determined by trypan blue staining.

RESULTS

Respiratory tract colonization of mice by WT and ΔCYA. Previously, we constructed a strain (ΔCYA) with an in-frame deletion of the *cyaA* gene encoding ACT (3). Six-week-old female BALB/c mice were intranasally inoculated with a high dose (3×10^6 CFU) or a low dose (5×10^4 CFU) of either WT or ΔCYA, and lower respiratory tract colonization was assessed 7 days later (around the peak of WT colonization). ΔCYA showed a significant defect in colonization (1 to 2 $\log_{10}$ reduction) at each dose (Fig. 1A). The time courses of lower respiratory tract colonization by these two strains in BALB/c mice were followed after inoculation with 2.5×10^5 CFU (an intermediate dose). WT showed a characteristic increase in colonization, with a peak at day 7 postinoculation, followed by gradual clearance of the infection over the next 2 weeks (Fig. 1B). In comparison, ΔCYA failed to multiply significantly and showed a defect in colonization from day 4 to day 14 (Fig. 1B). These data demonstrate that ACT plays an important role during infection that allows optimal colonization of the mouse lower respiratory tract by *B. pertussis*. The phenotype of ΔCYA is quite similar to that of ΔPT in this infection model (2).

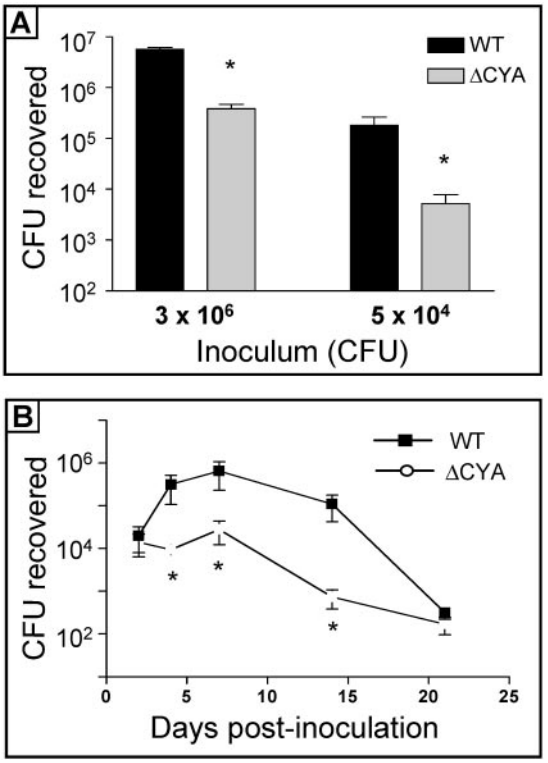

FIG. 1. (A) Groups of four to five BALB/c mice were inoculated with the indicated doses of either WT (darker bars) or ΔCYA (lighter bars), and colonization levels were assessed after 7 days. Results show a significant defect in colonization by ΔCYA at both doses. (B) Comparison of the time courses of respiratory tract colonization by WT (squares) and ΔCYA (open circles) after inoculation of BALB/c mice with 2.5×10^5 CFU of either strain. Each value is the mean ± standard deviation (SD) of results for groups of four to five mice. *, $P < 0.05$.

Mixed infection with WT and ΔCYA strains. Previously we found that ΔPT shows no competitive disadvantage in a mixed infection with WT, despite its colonization defect in a single-strain infection, and that it colonizes at higher levels in the mixed infection than in the single-strain infection (2). This is almost certainly due to the secreted soluble PT acting on the host to benefit all bacteria present in the infection. To determine whether ΔCYA demonstrates a similar phenotype when mice are coinfected with WT, we performed mouse infection experiments with inocula consisting of WT and ΔCYA mixed together in different ratios. Six-week-old female BALB/c mice were intranasally inoculated with 5×10^5 CFU of a mixture of WT and ΔCYA in ratios of approximately 5:1 and 1:5, and lower respiratory tract colonization and the ratio of the colonizing strains were determined on day 7 postinoculation. As shown in Fig. 2A, the overall colonization level was greater for the 5:1 ratio inoculum, presumably due to the higher number of WT bacteria in this inoculum. The ratio of the two strains in the inoculum and among the CFU recovered from each infection was determined by assessment of colony hemolytic phenotype (ΔCYA is nonhemolytic), and the results showed that the ratio of WT to ΔCYA recovered from each mouse was greater than the starting ratio in the inoculum (Fig. 2B). For the 5:1 ratio inoculum (82% WT), the mean percentage of WT colonies recovered from the mice was 96.5%, and for the 1:5

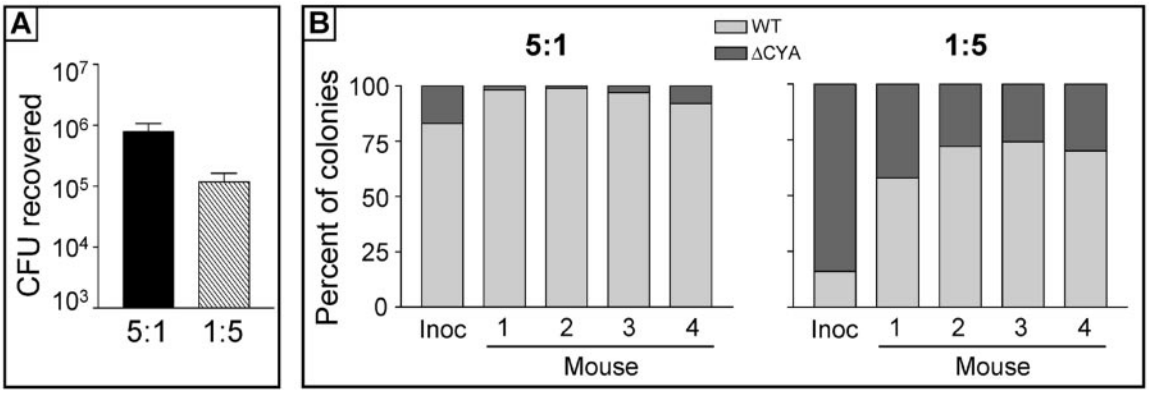

FIG. 2. Groups of four BALB/c mice were inoculated with 5×10^5 CFU of a mixture of WT and ΔCYA at the indicated ratios. (A) Colonization levels from each infection at 7 days postinoculation. Bars indicate means + SDs. (B) Ratios of the two strains in the inoculum (Inoc) or the CFU recovered from individual mice infected with each mixture, expressed as percentages.

ratio inoculum (16% WT), the mean percentage of WT colonies recovered from the mice was 68.5%. In addition, ΔCYA colonized at slightly lower levels in these mixed infections than in the single-strain infection. These results demonstrate that ΔCYA is at a significant competitive disadvantage versus WT in a mixed infection. This phenotype is in contrast to that of ΔPT and suggests that ACT acts more as a cell-associated factor than a soluble secreted toxin in its role in enhancing respiratory tract colonization.

Effect of coadministration of purified ACT on ΔCYA colonization. To test further the idea that ACT acts as a cell-associated factor benefiting only the producing bacteria to promote colonization, we performed mouse infection experiments in which we coadministered purified ACT mixed with the ΔCYA inoculum. Six-week-old female BALB/c mice were intranasally inoculated with either 3×10^5 CFU of WT or a mixture of 3×10^5 CFU of ΔCYA and 200 ng purified ACT (or buffer as a control), and lower respiratory tract colonization was assessed 4 days postinoculation. The results showed that coadministration of ACT had no beneficial effect on the colonization level of ΔCYA (Fig. 3). However, one possibility is that ACT plays a role at a later time after inoculation and that the ACT protein administered at the time of inoculation is cleared or inactivated by the time its function is needed by the bacteria. To test this possibility, mice were inoculated with 3×10^5 CFU of ΔCYA and then on days 1 or 2 postinoculation, groups of these mice were intranasally treated with either 200 ng purified ACT or with an equivalent volume of buffer as a control, and lower respiratory tract colonization was assessed 4 days postinoculation. The results showed that, similar to the coadministration of ACT with the bacteria, administration of ACT at the later time points had no beneficial effect on the colonization level of ΔCYA (Fig. 3). The slight increase in colonization in mice administered ACT 2 days postinoculation is not significantly different than the level in the buffer control group or the coadministration (day 0) group. The activity of the purified ACT was confirmed by its cytotoxicity for J774.1 macrophage cells (data not shown). These data suggest that, in contrast to PT, purified ACT cannot "complement" the colonization defect of the mutant strain, further supporting the idea that ACT acts in a cell-associated manner rather than as a soluble secreted protein.

Comparison of ΔPT and ΔCYA colonization early after inoculation. Previously, we found that ΔPT colonization was significantly lower than that of WT by day 2 postinoculation and that PT production by WT appeared to delay the influx of neutrophils to the lungs of infected mice (2). The observations that ΔCYA colonization was not significantly different than that of WT at day 2 postinoculation (Fig. 1B) and that purified ACT did not enhance ΔCYA colonization (Fig. 3) were both in contrast to the results obtained for ΔPT and PT (2) and led us to hypothesize that the two toxins may play different roles at different times during the early phase of *B. pertussis* infection. To test this idea we inoculated mice with 2×10^5 CFU of either WT, ΔPT, or ΔCYA and assessed colonization 3 h (day 0), 1 day, 2 days, and 4 days postinoculation. As shown in Fig. 4A, the colonization defect of ΔPT manifests earlier than that of ΔCYA, with a significant difference between the two mutant strains at day 2 postinoculation, although in this experiment, ΔCYA colonization was also significantly reduced compared to that of WT at day 2. By day 4 colonization levels of the two mutants were equivalent, and both were significantly lower

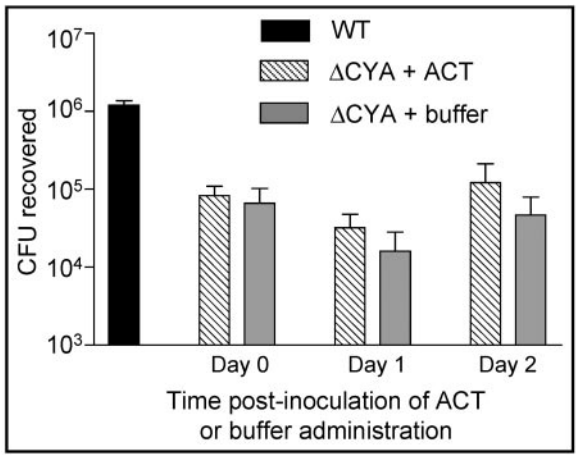

FIG. 3. BALB/c mice (four per group) were inoculated with approximately 3×10^5 CFU of WT (dark bar) or an equivalent dose of ΔCYA, with administration of 200 ng purified ACT or an equivalent volume of buffer on days 0, 1, and 2 postinoculation. Colonization levels at day 4 postinoculation are shown. Bars indicate means + SDs.

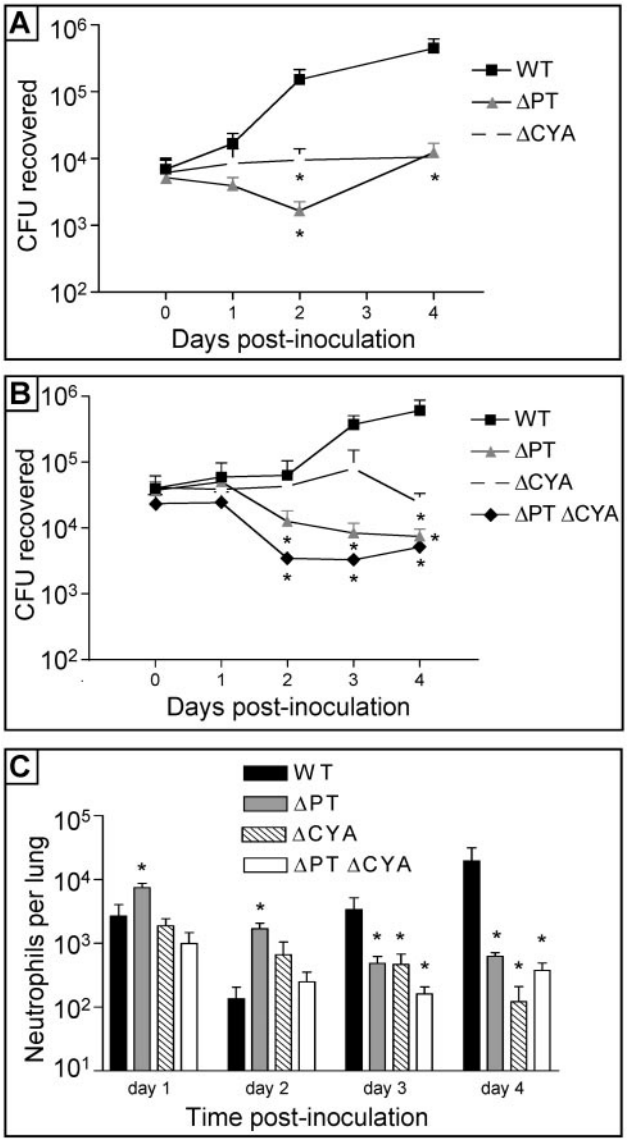

FIG. 4. (A) BALB/c mice were inoculated with approximately 2×10^5 CFU of WT, ΔPT, or ΔCYA, and colonization levels were assessed at the indicated times after inoculation (points indicate means + SDs of values from four mice). (B) BALB/c mice were inoculated with approximately 4×10^5 CFU of WT, ΔPT, ΔCYA, or ΔPT ΔCYA, and colonization levels were assessed at the indicated times after inoculation (points indicate means + SDs of values from four mice). (C) Neutrophil numbers in bronchoalveolar lavage fluid from mice in the experiment shown in panel B. *, $P < 0.05$.

(approximately 50-fold) than that of WT (Fig. 4A). The experiment was repeated, this time with the inclusion of the double mutant strain ΔPT ΔCYA, and in addition to assessment of colonization, we performed BAL on the same mice and analyzed the cell content to assess the level of neutrophil influx in the lungs. Mice were inoculated with 4×10^5 CFU of WT or one of the mutant strains, and as shown in Fig. 4B; the colonization results were similar to those in the first experiment, with ΔPT showing a significant defect earlier than ΔCYA. Not surprisingly, the double mutant strain was the most defective for colonization (Fig. 4B). Assessment of neutrophil influx in response to infection with each strain revealed a significantly higher level of neutrophils in the BAL fluid of ΔPT-infected mice than in that of ΔCYA-infected mice, versus the level in the BAL fluid of WT-infected mice on days 1 and 2 postinoculation (Fig. 4C). By day 3 postinoculation, the level of neutrophils in the BAL fluid of WT-infected mice was significantly higher than that in the mice infected with any other strain, with a further increase by day 4, presumably due to the higher colonization level achieved by WT at these time points. In contrast there was no increase in the number of neutrophils over time in response to infection with any of the mutant strains. The numbers of macrophages (approximately 10^4) in the BAL samples from these mice were not significantly different between strains and did not increase over the time course of this experiment (data not shown). From these data, we conclude that ACT, unlike PT, has no effect on early neutrophil influx into the lungs of *B. pertussis*-infected mice.

DISCUSSION

In this study we have shown that ACT plays an important role in lower respiratory tract infection by *B. pertussis* in our mouse model. A mutant with an in-frame deletion of *cyaA* (ΔCYA) was significantly defective in colonization at two different doses and over the time course of infection, at least between days 4 and 14 postinoculation. This is not surprising based on the similar phenotypes of various ACT-deficient mutants in other studies (7, 18), but the mouse models of infection used in those studies differ from ours, and we have obtained contrasting results on the phenotype of the ΔPT strain in our model to those reported using these other models (2).

Mixed infection with WT and ΔCYA strains revealed that, unlike ΔPT, ΔCYA is at a competitive disadvantage versus WT. This suggests that ACT acts more in a cell-associated manner than as a freely soluble toxin, benefiting the cells producing it rather than the bacterial population as a whole. ACT has been described as a cell-associated toxin (15), probably due to its interaction with filamentons hemagglutinin on the bacterial cell surface (34). However, a recent report demonstrated that newly secreted toxin, rather than cell-associated toxin, was responsible for the intoxication of cultured mammalian cells and that different *B. pertussis* strains with widely different levels of cell-associated ACT could intoxicate cells equally (9). Other data in that report suggested that the interaction of bacteria with cultured cells was important for efficient delivery of ACT (9). All together with our data from the mixed infections, the most plausible explanation is that during a *B. pertussis* infection, ACT acts on target cells in the close vicinity of the bacteria producing it, probably after binding of the bacteria to the target cell. In this case, the number of bacterial cells bound to each target mammalian cell must be relatively low; otherwise, the mixture of WT and ΔCYA cells bound to a target cell (in the mixed infection) would all benefit from the cell intoxication by ACT produced by the WT cells. This property of ACT distinguishes it from PT, which appears to act as a freely soluble toxin benefiting all bacteria during mixed infection (2) and suggests different specific roles for the two toxins during infection. The inability of purified exogenously added ACT to enhance *B. pertussis* colonization is consistent with this idea, since the freely soluble toxin may not act in the

appropriate spatial or temporal manner on the specific cells to which the bacteria are bound. However, this may also be due to the more trivial reason that ACT is relatively unstable in solution (once diluted out of the urea buffer) and may undergo aggregation into inactive complexes before reaching relevant target cells. With this in mind, we administered ACT on days 1 and 2 postinoculation, as well as coadministering ACT with the bacteria, in case ACT was needed only at these later time points of the infection, but still no enhancing effect was observed.

Our observation from the first time course infection experiment that ΔCYA did not show a significant colonization defect versus WT on day 2 postinoculation (Fig. 1B) led us to speculate that the timings of the requirement for the activities of ACT and PT early during *B. pertussis* infection may be slightly different, with PT perhaps being required earlier since ΔPT was significantly defective for colonization by day 2 (2). Our data from the early infection experiments (Fig. 4) indeed strongly suggest that PT plays a slightly earlier role in the infection than ACT, since the defect in ΔPT colonization was consistently manifested earlier than that of ΔCYA, although both mutants were equally defective by day 4 postinoculation. Neutrophil influx was greater in response to ΔPT infection than to WT infection at early time points, as we had previously observed, but this was not true for ΔCYA, suggesting that ACT does not act to inhibit neutrophil recruitment to the site of infection. Interestingly, neutrophil influx in response to WT infection greatly exceeded that in response to infection with any of the mutants by day 3 postinoculation, with a further increase by day 4. This may be due to the much higher numbers of colonizing bacteria by these time points, with the release of neutrophil-recruiting stimuli that overcome the effect of PT. We hypothesize that PT plays an important early role for *B. pertussis* infection by delaying the influx of neutrophils to the site of infection during the first 24 to 48 h postinoculation and that ACT then plays an important role in the intoxication of recruited neutrophils after the interaction of the bacteria with these cells. ACT is known to enter cells efficiently after binding to the CD11b/CD18 integrin receptor present on neutrophils (12) and to have deleterious effects on neutrophil activities (4, 5, 29), and in a study on the closely related pathogen *Bordetella bronchiseptica*, neutrophils were identified as the major target cells for ACT in promoting infection (14). Therefore, these toxins may provide a one-two punch on neutrophil recruitment and activity that is essential for optimal infection and colonization of the respiratory tract by *B. pertussis*.

ACKNOWLEDGMENTS

This work was supported by PHS grant AI50022.

We thank Gina Donato and Erik Hewlett for purified ACT and Zoë Worthington and Roger Plaut for critiquing the manuscript.

REFERENCES

1. **Brennan, M. J., J. L. David, J. G. Kenimer, and C. R. Manclark.** 1988. Lectin-like binding of pertussis toxin to a 165 kilodalton Chinese hamster ovary cell glycoprotein. J. Biol. Chem. **263:**4895–4899.
2. **Carbonetti, N. H., G. V. Artamonova, R. M. Mays, and Z. E. V. Worthington.** 2003. Pertussis toxin plays an early role in respiratory tract colonization by *Bordetella pertussis.* Infect. Immun. **71:**6358–6366.
3. **Carbonetti, N. H., G. V. Artamonova, C. Andreasen, E. Dudley, R. M. Mays, and Z. E. V. Worthington.** 2004. Suppression of serum antibody responses by pertussis toxin after respiratory tract colonization by *Bordetella pertussis* and identification of an immunodominant lipoprotein. Infect. Immun. **72:**3350–3358.
4. **Confer, D. L., and J. W. Eaton.** 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science **217:**948–950.
5. **Friedman, R. L., R. L. Fiederlein, L. Glasser, and J. N. Gagliani.** 1987. *Bordetella pertussis* adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. Infect. Immun. **55:**135–140.
6. **Glaser, P., A. Danchin, D. Ladant, O. Barzu, and A. Ullmann.** 1988. *Bordetella pertussis* adenylate cyclase: the gene and the protein. Tokai J. Exp. Clin. Med. **13:**239–252.
7. **Goodwin, M. S., and A. A. Weiss.** 1990. Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. Infect. Immun. **58:**3445–3447.
8. **Gray, M., G. Szabo, A. S. Otero, L. Gray, and E. Hewlett.** 1998. Distinct mechanisms for K+ efflux, intoxication, and hemolysis by *Bordetella pertussis* AC toxin. J. Biol. Chem. **273:**18260–18267.
9. **Gray, M. C., G. M. Donato, F. R. Jones, T. Kim, and E. L. Hewlett.** 2004. Newly secreted adenylate cyclase toxin is responsible for intoxication of target cells by *Bordetella pertussis.* Mol. Microbiol. **53:**1709–1719.
10. **Gross, M. K., D. C. Au, A. L. Smith, and D. R. Storm.** 1992. Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional *cya*A toxin of *Bordetella pertussis* abolish virulence. Proc. Natl. Acad. Sci. USA **89:**4898–4902.
11. **Gueirard, P., A. Druilhe, M. Pretolani, and N. Guiso.** 1998. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection in vivo. Infect. Immun. **66:**1718–1725.
12. **Guermonprez, P., N. Khelef, E. Blouin, P. Rieu, P. Ricciardi-Castagnoli, N. Guiso, D. Ladant, and C. Leclerc.** 2001. The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the $\alpha_M\beta_2$ integrin (CD11b/CD18). J. Exp. Med. **193:**1035–1044.
13. **Guiso, N., M. Rocancourt, M. Szatanik, and J. M. Alonso.** 1989. *Bordetella* adenylate cyclase is a virulence associated factor and an immunoprotective antigen. Microb. Pathog. **7:**373–380.
14. **Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller.** 1999. Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating host immunity. Infect. Immun. **67:**1493–1500.
15. **Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff.** 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis.* Proc. Natl. Acad. Sci. USA **73:**1926–1930.
16. **Kasuga, T., Y. Nakase, K. Ukishima, and K. Takatsu.** 1954. Studies on *Haemophilus pertussis.* Relation between the phase of bacilli and the progress of the whooping-cough. Kitasato Arch. Exp. Med. **27:**57–62.
17. **Katada, T., M. Tamura, and M. Ui.** 1983. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch. Biochem. Biophys. **224:**290–298.
18. **Khelef, N., H. Sakamoto, and N. Guiso.** 1992. Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb. Pathog. **12:**227–235.
19. **Khelef, N., A. Zychlinsky, and N. Guiso.** 1993. *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect. Immun. **61:**4064–4071.
20. **Martin, C., M. A. Requero, J. Masin, I. Konopasek, F. M. Goni, P. Sebo, and H. Ostolaza.** 2004. Membrane restructuring by *Bordetella pertussis* adenylate cyclase toxin, a member of the RTX toxin family. J. Bacteriol. **186:**3760–3765.
21. **Morse, S. I., and J. H. Morse.** 1976. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis.* J. Exp. Med. **143:**1483–1502.
22. **Moss, J., S. J. Stanley, D. L. Burns, J. A. Hsia, D. A. Yost, G. A. Myers, and E. L. Hewlett.** 1983. Activation by thiol of the latent NAD glycohydrolase and ADP-ribosyltransferase activities of *Bordetella pertussis* toxin (islet-activating protein). J. Biol. Chem. **258:**11879–11882.
23. **Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski.** 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis.* Infect. Immun. **33:**820–826.
24. **Pittman, M.** 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. Rev. Infect. Dis. **1:**401–412.
25. **Reisine, T.** 1990. Pertussis toxin in the analysis of receptor mechanisms. Biochem. Pharmacol. **39:**1499–1504.
26. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
27. **Szabo, G., M. C. Gray, and E. L. Hewlett.** 1994. Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium- and polarity-dependent manner. J. Biol. Chem. **269:**22496–22499.
28. **Weingart, C. L., P. S. Mobberley-Schuman, E. L. Hewlett, M. C. Gray, and A. A. Weiss.** 2000. Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. Infect. Immun. **68:**7152–7155.

29. **Weingart, C. L., and A. A. Weiss.** 2000. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. Infect. Immun. **68:**1735–1739.
30. **Weiss, A. A., and M. S. Goodwin.** 1989. Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. Infect. Immun. **57:**3757–3764.
31. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis.* J. Infect. Dis. **150:**219–222.
32. **Witvliet, M. H., D. L. Burns, M. J. Brennan, J. T. Poolman, and C. R. Manclark.** 1989. Binding of pertussis toxin to eukaryotic cells and glycoproteins. Infect. Immun. **57:**3324–3330.
33. **Wolff, J., G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz.** 1980. Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. USA **77:**3841–3844.
34. **Zaretsky, F. R., M. C. Gray, and E. L. Hewlett.** 2002. Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin-filamentous haemagglutinin interaction. Mol. Microbiol. **45:**1589–1598.

Editor: A. D. O'Brien